Dear Dr. Krishnan,

First, we would like to take this opportunity to thank you, the Editors and the Reviewers for taking the time to carefully read the manuscript and for providing constructive suggestions and comments. We present our point-by-point response to the concerns raised by the Editors and Reviewers below. It is important to note that line numbers listed in this letter correspond to "Track Changes" turned off.

Thank you for your consideration.

With best wishes,
Arek Kulczyk and Megan DiIorio

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have carefully proofread the manuscript, and corrected all spelling and grammar mistakes. Changes are documented in the revised manuscript; these changes can be visualized by turning the "Track Changes" button on under "Review" tab in MS Word.

2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have removed personal pronouns from the text.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. All commercial products should be sufficiently referenced in the Table of Materials.

We have removed all company names from the text and revised the Table of Materials.

Are cryoSPARC, RELION and Scipion free/ open source software packages?

We have included the following statement in the text (lines 88-90): In this article, cryoSPARC v3, RELION-3, and Scipion 3 were used to obtain a high-resolution 3D reconstruction of AAV, a widely used vector for gene therapy[16]. Aforementioned software packages are free to academic users; cryoSPARC v3 and Scipion 3 require licenses.

JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of " cryoSPARC, RELION and Scipion " within your text. The

term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language.

We have reduced the number of instances of "cryoSPARC, RELION and Scipion" where appropriate.

4. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

We have moved the following sections from the Protocol to the Discussion: notes from section 3: lines 146 – 150 and lines 157 – 163 have been moved to lines 551 – 556 and 557 – 562, respectively. The note from section 4: lines 198 -202 has been moved to lines 566 – 570.

5. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

All steps in the protocol contain a maximum of 3 actions and 4 sentences per step.

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have revised the Protocol according to the above recommendations and added more details and/or references to the following highlighted steps: step 6.5 (lines 233 – 236), step 6.6 (lines 238 -241), step 9.1 (lines 319 – 324), and step 12.2 (lines 384 – 387).

7. Line 121: What was the sample used in this study? Where was the data extracted from? Was the data generated in the authors lab?

We have added the following statement to the text (lines 95 - 99): Data were acquired at Oregon Health and Science University (OHSU) in Portland using a 300 kV Titan Krios electron microscope equipped with a Falcon 3 direct electron detector. Images were collected in a counting mode with a total dose of 28.38 $e^{-}/Å^{2}$ fractioned across 129 frames, and defocus range from -0.5 µm to -2.5 µm, at a pixel size of 1.045 Å using EPU. The sample of AAV-DJ was provided by the staff of OHSU.

8. Please ensure that the highlighted steps (not exceeding 3 pages) form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences

(not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in the imperative tense.

We have revised the highlighted steps. These steps form a cohesive narrative, contain complete sentences, at least one action item, and do not exceed 3 pages.

9. As we are a methods journal, please ensure that the Discussion explicitly covers the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol

Critical steps of the Protocol are described in paragraphs 1, 3 and 4 of the Discussion.

b) Any modifications and troubleshooting of the technique

Please see paragraphs 4 and 5.

c) Any limitations of the technique

Please see a paragraph 2.

d) The significance with respect to existing methods

Please see a paragraph 6.

e) Any future applications of the technique

Please see paragraphs 1 and 6.

10. Please do not use the &-sign or the word “and” when listing authors in the references. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc.

We have revised and corrected the references.

11. Figure 2/3/5: Please include scale bars in all the images of the panel.

We have included scale bars in all relevant images from Figures 2, 3, 4, 5 and 7.

12. Please ensure that the Table of Materials includes all the supplies (reagents, chemicals, instruments, equipment, software, etc.) used in the study. Please sort the table in alphabetical order.

We have updated the Table of Materials and alphabetically ordered all listed items.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors described a cryo-EM single-particle reconstruction workflow integrated with Relion, cryosparc2, and Scipion. Combining different processing software is a common way for maximizing the strengths of all the software.

Major Concerns:

1. The authors should document better the dataset used and shown in the paper. For example, how the data collection was done. If the authors do not collect the dataset, please state where the dataset is collected. For instance, if the data is collected in counting mode or super-resolution mode, K2 camera? K3 camera? Falcon camera? Energy filter used or not? Defocus range? Without this information about the dataset, the reader can't follow their processing steps.

We apologize for the omission of this important information in the initial manuscript. We have added the following statement to the text in a revised manuscript (lines 95 - 99): Data was acquired at Oregon Health and Science University (OHSU) in Portland using a 300 kV Titan Krios electron microscope equipped with a Falcon 3 direct electron detector. Images were collected in a counting mode with a total dose of 28.38 $e^{-}/Å^{2}$ fractioned across 129 frames, and defocus range from -0.5 µm to -2.5 µm, at a pixel size of 1.045 Å using EPU. The sample of AAV-DJ was provided by the staff of OHSU.

2. "0.22 e/A^2 total exposure dose" must be a typo. Otherwise, it is hard to believe the authors could achieve a 3-A resolution.

Thank you for identifying this typo. A total dose was 28.38 $e^{-}/Å^{2}$. Each movie stack contained 129 frames acquired with a dose of 0.22 $e^{-}/Å^{2}$ per frame.

3. The features shown in the figure do not match my expectation from a 2.1-A map. Where are the water molecules? Where are the ions? Is the map sharpened? Would you please show a figure how the improvement from 2.9 to 2.1-A looks like? Xmipp-highres from Scipion processing is documented in JSB 2018 paper; the FSC may not be the best way to show the map's resolution. Because all the FSC curves somehow do not reach 0 at the Nyquist frequency, which usually means the same averaging unit is being used multiple times in the reconstruction. But I am not sure if this is also the case here. I suggest the author show the expected features at this resolution to convince the readers.

We would like to thank the Reviewer for this constructive comment. We have re-analyzed the data and following a careful inspection of the map, we concluded that a more adequate resolution estimate for the map is 2.3 Å. We do agree with the Reviewer that reporting the resolution estimate according to a single criterion (e.g. 0.143 criterion) from the FSC curve may not be the best way to reflect the resolution of the map as described in Sorzano, C. O. S. *et al.* A

new algorithm for high-resolution reconstruction of single particles by electron microscopy. *Journal of Structural Biology.* 204 (2), 329-337, (2018). Because resolution may vary from point to point in the map, it is often more appropriate to present distribution of local resolution estimates. Thus, following the Reviewer's recommendation, we re-analyzed the 2.9 Å and 2.3 Å maps using Xmipp – MonoRes. Results of the analysis are presented in Figure 7 and discussed in paragraph 3 of the Representative Results (lines 458-473). Figure 7A-D clearly demonstrate the incremental improvement of the map during refinement when comparing local resolution distributions obtained for 2.9 Å and 2.3 Å maps.

Below we address other specific points made by the Reviewer. Densities representing water molecules and magnesium ions are presented in Figure 5D. We identified these densities by overlaying the 2.3 Å map with previously deposited atomic coordinates of AAV-DJ (PDB ID: 7fkr). The map was sharpened in Phenix using a B factor of -43.43. The FSC curves obtained in cryoSPARC and RELION do reach 0 at Nyquist frequency. However, we agree with the Reviewer that the way these plots were presented in Figure 6 were misleading. Therefore, we have redrawn plots in the figure. The FSC plot from Xmipp – highres does not reach 0 at Nyquist. In our opinion, this result suggests the resolution estimate is limited due to insufficient sampling during data collection as described in Penczek, P. A., Resolution Measures in Molecular Electron Microscopy. 482, 1-33 (2010). Interestingly, an analogous sample of AAV-DJ analyzed in the same cryo-EM center where we collected data was refined to 1.56 Å resolution (Xie, Q., Yoshioka, C. K., Chapman, M. S. Adeno-Associated Virus (AAV-DJ)-Cryo-EM Structure at 1.56 A Resolution. *Viruses*, *12* (10), (2020)). However, the above data set was acquired at 0.514 Å/px. In contrast, our data set was acquired at 1.045 Å/px.

Taken together, although the FSC curve calculated with Xmipp3 – highres indicates the Nyquist limit has been reached, MonRes analysis presented in Figure 7, along with a careful analysis of the EM map and map fitting with atomic coordinates of AAV presented in Figure 5 suggest an adequate resolution estimate for the map is 2.3 Å. Interestingly, similar discrepancy in Xmipp – highres and Xmipp – MonoRes estimates have been reported earlier, for example in Sorzano, C. O. S. *et al.* A new algorithm for high-resolution reconstruction of single particles by electron microscopy. *Journal of Structural Biology.* 204 (2), 329-337, (2018), and Jimenez-Moreno, A. *et al.* Cryo-EM and Single-Particle Analysis with Scipion 3. *Journal of Visualized Experiments.* (171), e62261, (2021).

4. If the mask is too tight, the reported resolution will be the Nyquist resolution. Maybe also show the mask and make sure the mask is not too tight.

A circular mask with a radius of 150 pixels was automatically applied by Xmipp3 – highres during refinements. We have used Chimera to create a mask for Xmipp–MonoRes analysis. This mask overlapped with the 2.3 Å and 2.9 Å reconstructions is presented in Figure 7D.

5. It would be great if the authors show monores slice of both 2.9 and 2.1-A maps.

The Xmipp – MonoRes slices along with local resolution histograms obtained for 2.3 Å and 2.9 Å maps are presented in Figure 7 A-B and Figure 7C, respectively.

6. In Figure 4. Flow chart - 2D classification results are both from cryosparc2. It makes more sense if the authors show representative 2D classification results from relion for 2D classification performed in Relion. Otherwise, it isn't clear.

We apologize for this mistake. Figure 4 has been revised accordingly to the Reviewer's comment.

7. The authors did not mention non-uniform refinement, heterogenous refinement, local motion correction in cryosparc2, and they should be removed from the flowchart.

The above-listed cryoSPARC protocols have been removed from the flowchart presented in Figure 1.

Reviewer #2:
Manuscript Summary:
The authors introduce an interesting image processing workflow for Single Particle Analysis by Cryo-Electron Microscopy combining multiple popular software suites (CryoSparc, Relion, and Scipion/Xmipp). The manuscript is easy to follow and its results support the validity of the approach. It will be a useful contribution for many practitioners.

Major Concerns:
None

Minor Concerns:
Scipion offers all the processes used in the article from CryoSparc and Relion. Would not have been more advantageous to perform the whole analysis within a single platform so that their results could also have been compared? At the moment, they are simply transferred from one package to the next without any quantitative comparison. The authors may want to comment on this possibility.

We thank Reviewer for this comment. Although, Scipion provides an integrative Python shell supporting algorithms from multiple platforms including cryoSPARC and RELION, the most recent implementations of these programs are not immediately available in Scipion. For instance, as far as we know, only RELION-3 offers Ewald Sphere Curvature Correction through the script Relion_reconstruct. Currently, there is no universal SPA platform accepted by the field. We think Scipion, along with Appion could become such platforms in the near future. Analysis of cryo-EM structures deposited in the PDB in recent years indicate the majority of these structures have been determined using RELION, whereas a number of cryoSPARC depositions is rapidly growing. We think it is important to reiterate the usefulness and application of Scipion, as its multitude of algorithms often yield the higher resolution reconstructions than RELION and cryoSPARC, as evidenced in this manuscript. Finally, we do

recognize the advantage of Scipion for quantitative comparison of reconstructions obtained using different SPA platforms. We have compared maps obtained in cryoSPARC and Scipion using Xmipp–MonoRes. Results of the analysis are presented in Figure 7 and discussed in paragraph 3 of the Representative Results (lines 458-473) in the revised manuscript.

As a minor remark, the beginning of the protocol is currently labelled as "Protocal" instead of "Protocol".

We have corrected the typo.

Reviewer #3:
Manuscript Summary:
This manuscript details processing single particle cryo-EM data to yield high resolution 3D structures with varying programs. While there are tutorials available for individual software packages, this demonstrates how to move data between 3 different packages and utilize different aspects of each to arrive at an improved structure. While this 3 method step may not be commonplace (it also may be more utilized than I am aware of), demonstrating using multiple platforms and the application of PyEM is a very useful tutorial that is an excellent tool for anyone learning to process this type of data. The written steps are easy to understand and good notes are supplied for the test sample specific parameters. This is a very welcome addition to the growing number of online tutorials for cryo-EM, from sample prep through data processing. Thank you!

We thank the Reviewer for this comment.

Major Concerns:
Including times estimates or the actual times required for the test dataset will be helpful to novices who may underestimate the time required to run these processes. Supply some reasoning behind why using certain packages for some steps is better than others. Steps 6.3 and 6.5 could use more detail.

Time estimates have been included in the flowchart presented in the Figure 1. Paragraph 6 in the Discussion now contains a section concerned with comparing algorithms from cryoSPARC, RELION and Scipion (lines 603-615). Step 6 of the Protocol has been re-written, and former sections 6.3 and 6.5 expanded in a revised manuscript as 6.1-6.4 and 6.8-6.9, respectively.

Minor Concerns:
Clearly indicate which version of softwares are being used. Describe what may be needed to access these programs, i.e. cryoSPARC is free for academic use but requires a license.

We have clearly indicated specific versions of all programs used in this study and added the following statement to the text (lines 88-90): In this article, cryoSPARC v3, RELION-3, and Scipion 3 were used to obtain a high-resolution 3D reconstruction of AAV, a widely used vector

for gene therapy[16]. Aforementioned software packages are free to academic users; cryoSPARC v3 and Scipion 3 require licenses.

Reviewer #4:
Manuscript Summary:
In the manuscript "A robust single-particle cryo-Electron Microscopy (cryo-EM) processing workflow with cryoSPARC, RELION and Scipion." Dilorio and Kulczyk provide a valuable workflow for routine structure determination through cryo-EM.

In the Cryo.EM field, every researcher has their own ways to obtain high-resolution data in a streamlined and straightforward manner. Therefore, it is difficult for me to review this methods paper or even provide further suggestions on improving it, as I am using a different approach that may not be compatible with intermediate steps as presented here. Nevertheless, overviews, as presented here, are still valuable providing alternative perspectives and generating new ideas. The manuscript is well organized and easy to follow. Therefore, I do not feel comfortable suggesting alternative strategies or enforcing significant modifications.

Minor Concerns:
However, something is horribly wrong with the FSC curves presented in Figure 7 - maybe the authors can explain why the FSC curve obtained from Scipion never reaches zero if this is not an artifact of some sort; the authors have to explain why this happens in the manuscript.

We have redrawn plots in the Figure 6. The FSC curves obtained in cryoSPARC and RELION do reach 0 at Nyquist frequency. However, the FSC plot from Xmipp – highres does not reach 0 at Nyquist indicating the resolution estimate is limited due to insufficient sampling during data collection as described in Penczek, P. A., Resolution Measures in Molecular Electron Microscopy. 482, 1-33 (2010). Similar behavior of the FSC plots obtained with Xmipp – highres have been observed earlier, for example in Sorzano, C. O. S. *et al.* A new algorithm for high-resolution reconstruction of single particles by electron microscopy. *Journal of Structural Biology.* 204 (2), 329-337, (2018), and Jimenez-Moreno, A. *et al.* Cryo-EM and Single-Particle Analysis with Scipion 3. *Journal of Visualized Experiments.* (171), e62261, (2021). Appropriate explanation has been included in the Representative Results section (lines 462-468) and in the legend of Figure 6.